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Evaluation of Colony Blot Hybridization for Characterization and Identification of *Nitrospira Sp.* in Wastewater

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Wastewater purification requires the removal of phosphate and nitrogen from the water samples before it can be returned to the ocean. The conversion of nitrite to nitrate is done in most part by *Nitrospira sp*, which are the second most abundant organisms found after *Nitrobacter sp.* DNA from a bioreactor simulating wastewater was used to isolate DNA, which was amplified using generic bacterial primers 27-F and 1542-R. The product was then ligated into PCR 2.1 vector and transformed into TOP10 cells. The transformed cells were screened using Ntpsa685. Ntspa685 is a probe specific for Nitrospira moscoviensis and Nitrospira marina and was used to detect positive clones using colony blot hybridization. The positive clones were digested with restriction enzymes for verification of an insert, and then sequenced. It was found that a very strong and large signal was generated on the nitrocellulose membrane containing the colonies; however, it was difficult to differentiate which colonies the signal had arisen from due to smearing. Of the five plasmids isolated and purified, only 1 contained an insert, and when sequenced; was an 88% match for *Nitrosococcus mobilis* and *Nitrosomas europaea*. It can be concluded that further experimentation would be required in order to optimize the colony blot hybridization parameters before it can be determined whether or not it would be a viable method used for high throughput screening.

In large compact urbans areas, there are sewage treatment plants that process wastewater before it can be returned to the water system. Sludge, which is also known as waste water, contains phosphate and nitrogen, which must be removed before it can be returned to the environment (4). Previous research has established that there are bacteria and other organisms that can be used for the removal of phosphate and nitrogen. However, researchers are discovering that there are more bacteria which have the ability to convert phosphate and nitrogen than previously thought. One of the latest methods being used for the purification of wastewater is to use biofilms. Biofilms contain a large assortment of nitrifying bacteria that can consist of ammonium-oxidizing bacteria and nitrite-oxidizing bacteria (5).

Nitrospira sp. are known to convert nitrite to nitrate, in the conversion of nitrite back into nitrogen. (5) Nitrospira sp. are believed to be the second most abundant species in a biofilm; and can be experimentally detected using DNA probes which are specific to the 16s ribosomal RNA gene (3). In previous experiments, researchers have examined the diversity in species and have used probes to determine which species of bacterium are related. It has been seen with the probe, Ntspa 685, that bacteria species which do not seem related in a phylogenic tree can be detected with the same probe (7,9). It has also been demonstrated that the probe, Ntspa 685, will only detect N. moscoviensis, and N. marina (4).

Colony blot hybridization is a high throughput method of screening a large number of individual colonies at one time. Colonies are grown and lysed directly on a nitrocellulose membrane. At this point, the membrane is then probed, with a labeled probe, and the signal captured on X-ray film(8).

In this experiment, DNA isolated from wastewater was amplified using primers, 27-F and 1542-R. (10) The product was then ligated into TOPO pCR2.1 plasmid, and transformed into TOP 10 cells. The transformed colonies were screened for the colonies containing an insert. These colonies were grown for colony blot hybridization. Further analysis of the colonies was done by restriction enzyme digests and sequencing. The purpose of this experiment was to determine if colony blot hybridization could be used as a reliable means of identifying potential positives clones.

MATERIALS AND METHODS

DNA Extraction. 1 mL of activated sludge from a bioreactor (Engineering department, UBC) was spun down in a microcentrifuge and resuspended with 0.9mL TEN homogenization buffer (0.1 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0). The cells in solution were lysed using mini-bead beating (mini beadbeater-1, Biospecs). DNA was extracted as previously described (1) with a slight modification in the protocol: a tris-buffered phenol:chloroform:isoamyl alcohol (24:24:1) extraction step was added after the TE-buffered phenol step. DNA was precipitated with two volumes of 95% ethanol and 1/10 volume of 3M sodium acetate (pH 5.5) overnight at -20°C. The precipitated nucleic acids were

washed with 70% ethanol and dried using a vacuum centrifuge. The nucleic acid was resuspended in 200µL of TE(10mM Tris-HCl, 1mM EDTA, pH 7.5). The amount of DNA was determined by A_{260} using a spectrophotometer.

PCR Amplification and TOPO Cloning. The extracted DNA was amplified using universal bacterial primers 27-F [5'-GAGTTTGATCCTGGCTCAG-3', position 9 to 27] and 1542-R [5'-AGAAAGGAGGTGATCCAGCC-3', position 1525 to 1542] to amplify the 16s RNA gene. The following reaction mixture was used: 50pmol of each primer, 1 µL of 25mM dNTP mixture, 5 µL of 10x PCR buffer containing $MgCl_2$, 0.25 µL of Taq DNA polymerase(5U/uL) (Invitrogen), 2µL of 1000-fold diluted DNA and sterile distilled water was added to a volume of 50µL. Amplification occurred under these conditions: 94ºC for 3 minutes, and then 94 ºC for 1 minute, 55 ºC for 1 minute and 72 ºC for 1 minute for 34 cycles. The final extension occurred at 72 ºC for 5 minutes. The amplified product with the least amount of non-specific amplification was directly ligated into the vector using the manufacturer's directions in the TOPO 2.1 kit and then transformed into chemically competent TOP10' cells.

Colony Hybridization. Colonies believed to contain inserts were duplicately grided onto agar plates containing ampicillin (50mg/mL) and one of which contained a nitrocellulose filter. The colonies were grown overnight at 37ºC(9). The cells on the nitrocellulose membrane were lysed with 0.5N NaOH. 0.5N NaOH was replaced by 1M Tris-Cl. Finally, the membrane was neutralized using 1.5M NaCl, 0.5M Tris-Cl solution. The membrane was left to dry at room temperature for 30 minutes before being heat-fixed in an 80°C oven for 2 hours. The nitrocellulose membrane was blocked with 1% western blocking reagent (Roche, catalog # 112811300) and subsequently washed in 5mL of PBS + 0.5% Tween (8g of NaCl,0.2g of KCl,1.44g of Na_2HPO_4, 0.24g of KH_2PO_4, 1L distilled water, 0.5mL Tween-20, pH 7.4) solution. The probe, Ntspa685 (800ng/mL, stock concentration=1052µg/mL), was heated @ 95°C for 5 minutes, cooled on ice, and added to hybridization buffer (25mL 20x SSC, 20mL blocking solution, 1mL 10% N-laurylsacrosine, 0.2mL 10% SDS, 50mL formamide, and 3.8mL distilled water) and incubated overnight with the nitrocellulose membrane at 60°C (10). Two 5 minute washes with 2x wash buffer (10), and 2 subsequent washes in 0.1x wash buffer for 15 minutes. Lastly, the membrane was rinsed in maleic acid solution for 5 minutes. The membrane was treated with 1% blocking solution overnight at 4°C to prevent non-specific binding of the probe.

Colonies were detected using streptavidin-POD diluted in 1% blocking solution (0.5µL streptavidin-POD, 12.5µL 1% blocking solution). The excess unbound streptavidin-POD was removed by washing four times with a solution of maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl; adjust to pH 7.5 with NaOH) containing 0.3% Tween-20 for 15 minutes.

Visualization of Positive Colonies. The positive colonies on the membrane were detected using the Roche Lumi-light system (catalog # 12015200001). Three milliliters of reagent A and reagent B were mixed together, applied to the membrane, and incubated for 10 minutes at room temperature. Excess reagent was drained off, and the membrane was placed in Saran-wrap before being placed into the film cassette (sample side up), topped with a sheet of X-ray film and exposed for 2 seconds since the fluorescence was extremely strong.

Restriction Enzyme Digest. Positive colonies from the colony hybridization were grown overnight in LB broth containing 100mg/mL of ampicillin. The plasmid was extracted using the Fermentas Plasmid mini-prep kit (Cat #K0501) with a few minor changes. LB broth (2.5 mL) was used for each column, and the plasmid was eluted with 100µL of elution buffer instead of 50µL. The purified plasmids were digested with EcoR1, Alu1 or EcoR1 and Alu1 together. For a single digest, 250ng of the purified plasmid was added to 2µL of 10x REact1 digest buffer, 1µL of the restriction enzyme, and distilled water was added to a final volume of 20µL. For the double digest containing EcoR1 and Alu1, 1µL of each restriction enzyme was added into the restriction digest containing 250ng of purified plasmid, 2µL of 10x REact1 digest buffer, and distilled water – to a final volume of 20µL. The restriction enzyme digest was incubated at 37°C for 2 hours. Reactions were stopped by denaturing at 65°C for 10 minutes. The digested plasmids were run on a 2% agarose gel to determine whether a 1.5kb 16s RNA gene product was inserted into the TOPO vector. The isolated and purified plasmids were then sent for sequencing (Nucleic Acid Protein Service Unit, Michael Smith Labs at UBC).

RESULTS

DNA Extraction and PCR Amplification. 1mL of bioreactor sludge yielded 9.26 µg of DNA but the absorbance values showed a peak at 270nm rather than 260nm. The DNA concentration may be overestimated due to contamination. Purified bioreactor DNA could not be amplified under regular conditions due to inhibitors. The DNA was diluted to dilute out the inhibitors using a 10, 100 and a 1000 fold dilution with sterile distilled water. A 1:100 or 1:1000 dilution was required in order to get DNA amplification, with the 1:1000 dilution producing the product containing the least amount of non-specific binding (Figure 1).

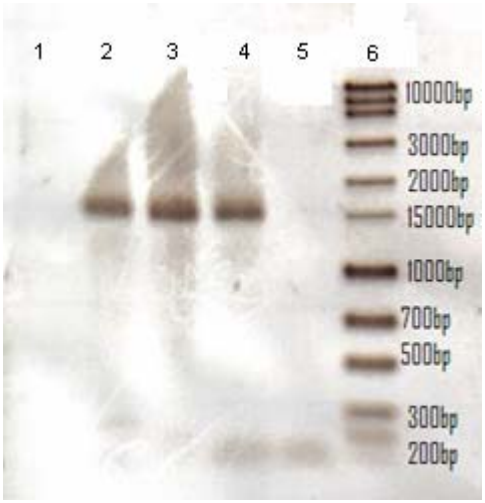

Fig 1. Effect of dilution on the potential to form PCR products for 16s RNA. Extracted DNA sample was diluted (1/10 (lane 2), 1/100 (lane 3), 1/1000 (lane 4)), amplified by PCR and separated on an agarose gel. A 1/1000 dilution showed the least amount of product however it also contained the least amount of non-specific binding. Lane 1 contains undiluted sample. Lane 5 is the negative control. Lane 6 is a standard DNA ladder (Fermentas, SM1283).

Colony Hybridization. The 24 transformed TOP10 colonies were grown on a nitrocellulose membrane on LB agar containing 50mg/mL of ampicillin with a colony of DH5α containing the plasmid pBR322 as a negative control. (8) When the plates were examined the following day, it was noticed that the nitrocellulose membrane was relatively damp and remained adherent to the agar. It was found that a majority of the colonies placed into the nitrocellulose membrane fluoresced when probed with Ntspa685 (Figure 2). It was seen that the quality of the signal was very strong,

however, it was difficult to locate definite edges to each colony, and it was seen that some of the colonies ended up smeared together making it difficult to differentiate which colony generated the signal. It was also seen that there was a significant amount of background that was seen on the X-ray film.

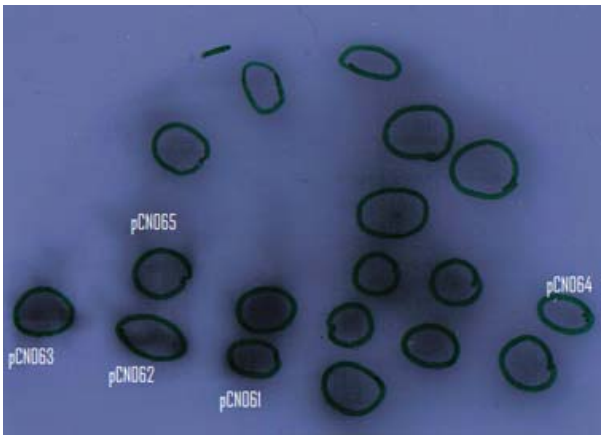

Fig 2. X-ray film after 2 second exposure to the colony blot where Ntspa685 probe is detected using chemiluminescence. pCN065 was the only colony containing an insert; however the colony's fluorescence was not as bright as the other colonies.

Plasmid Isolation. It was found that the Fermentas mini-prep kit generated a high yield of purified plasmid, with the lowest amount from plasmid pCN064 having 7.56 µg, and a highest yield from plasmid pCN065 with 14.03 µg (Table 1). There is no correspondence between the size of the colony, the relative fluorescence of the colony during chemiluminescence and the amount of plasmid isolated.

Table 1: The amount of plasmid isolated from the five selected colonies which emitted a strong signal when probed with Ntspa685.

Plasmid	Amount Isolated (µg)
pCN061	14.22
pCN062	11.91
pCN063	9.02
pCN064	7.56
pCN065	14.03

Restriction Enzyme Digest and Sequencing. Of the 5 plasmids purified using the Fermentas Microprep kit, pCN061, pCN062, pCN063, and pCN064 all did not contain an insert. A restriction digest done for CN06-005 with EcoR1 produced 2 fragments, a 850bp fragment, and a 700bp fragment (Figure 3). Further comparison with Alu1 could not be made due to the numerous Alu1 cut sites within pCR2.1. However, the bands in pCN065 correspond to the theorized bands of the *Nitrospira sp.* 16s RNA gene when digested with EcoR1, suggesting a potential match; thus, was sent off to be sequenced. Two other plasmids, pCN063and pCN064, were also sent off to be sequenced. pCN063 contained a very strong band at 350bp and pCN064 was used as the representative of pCN061, pCN062 and pCN064.

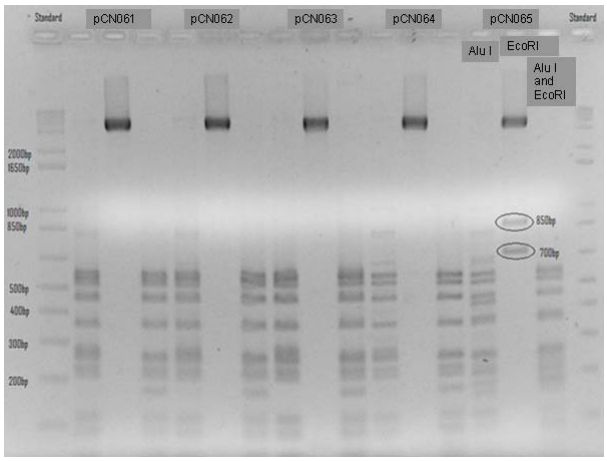

Fig 3. EcoR1 and Acu1 digest of the probed clones to assess the presence of potential 16s RNA inserts. Each plasmid was digested with Alu1 in the first sample, EcoR1 for the second sample, and EcoR1 and Alu1 in the third sample.

The sequenced result from pCN065 was analyzed using LaserGene software (DNAstar Inc., Madison, WI, USA) and compared with the NCBI database using Megablast. It was seen that the closest possible match to CN06-005 was the 16s ribosomal RNA gene of *Nitrosococcus mobilis* and *Nitrosomonas europaea*.

DISCUSSION

Colony blot hybridization was used as a method to screen for transformants containing a 16S RNA gene inserts. A majority of the colonies directly spotted onto the nitrocellulose membrane, were believed to be potential positives; however, several visible colonies, which had a blue centre and a white perimeter, and therefore thought not to contain an insert were also spotted onto the membrane along with the negative control, pBR322. A majority of the colonies fluoresced when probed with Ntspa 685 including the negative control containing a pBR322 plasmid. The colonies thought not to contain an insert did not show up under chemiluminescence.

In the preparation of the colonies for hybridization, the cells were grided onto agar plate. After an overnight incubation, the diameter of the colonies ranged from 5-10mm. According to Hu, the colonies for a colony blot should be 2-3mm after 12 to 24 hour incubation (ref). Using the 2 – 3 mm colonies, Hu

found that 75% of all the positive colonies bound onto the membrane were detected. Since the colonies used in the experiment were about 3 times larger in size, the overgrown colonies could have interfered with the ability to detect the potential positives. It has been published that overgrown colonies can lead to an interfering background, making it difficult to distinguish the fluorescing colonies. In order to prevent the variation in size of the colonies, the streaking loop should be touched lightly to the surface of the membrane instead of lightly rubbing it onto the nitrocellulose membrane. Another possibility is to use the tip of a sterile innoculating needle to lightly spot the colonies to ensure that they are of even size.

The colonies were grown on an agar plate with a nitrocellulose membrane placed on top. The nitrocellulose membrane adhered to the agar plate due to the light layer of water that kept the plates moist. The damp plate could account for the movement of the bacteria along the surface of the nitrocellulose membrane, which would account for the fuzzy edges seen on the X-ray film. To prevent the smearing of the colonies, and the movement of the bacteria on the plate, a semi-dry agar plate containing ampillicin should be used.

Another possibility which could have led to false positives could be due to the cellular debris left behind after the cells were lysed. Since the cells are lysed directly on the membrane, the proteins, RNA and other cellular material could have remained on the surface of the nitrocellulose membrane, and become bound to the membrane. This could have caused difficulty in the analysis of the colony blot, as the signal generated under chemiluminescence could have arisen due to RNA-DNA, DNA-DNA, or even protein-DNA binding, and is undistinguishable.

Only one of the five plasmids contained an insert. Further analysis of the TOPO pCR2.1 plasmid sequence was performed to determine if the Ntspa685 probe could have potentially been bound to the plasmid during nucleic acid hybridization. Comparison of the plasmid sequence with the Ntspa685 sequence using LaserGene showed the possibly of a match if the hybridization was done under less stringent conditions. The Ntspa685 probe contains several 3bp regions which are directly aligned with the pCR2.1 plasmid sequence as compared to the 16s RNA gene, which contained a 1 large matching region, and several smaller ones. There is about a 40% similarity between the probe and the plasmid; however, this is in groups of 3bp regions, which may or may not allow the probe to bind. Yu (ref) has determined that the melting temperature of the probe, Ntspa685 is 63°C; therefore, if the temperature of the hybridization washes were increased from 60°C to 63°C it would increase the stringency of the probe, and minimize the number of false positives (8).

It was seen that the insert obtained in pCN065 had the greatest similarity to *Nitrosococcus mobilis*, and *Nitrosomonas europaea,* even though *Nitrospira sp*. are the second most abundant species found in a wastewater sample. Since the sample used had arisen from a bioreactor, and the conditions within the bioreactor are not known, the relative abundances found in nature may not apply to that of the bioreactor. It is possible that abundance of *Nitrospira sp*. was decreased and *Nitrosococcus mobilis* growth conditions were favoured in the bioreactor allowing for a higher abundance. It is possible that the relative amounts of oxygen found in the bioreactor favours that of *Nitrosococcus mobilis* instead of *Nitrospira sp.,* which could account for the identification of *Nitrosococcus mobilis* using the Ntspa685.

It can be concluded that further experimentation would be required in order to optimize the colony blot hybridization parameters before it can be determined whether or not it would be a viable method used for high throughput screening.

FUTURE EXPERIMENTS

In order to be able to use colony blot hybridization as a reliable means of screening, further experimentation is required to determine whether the size of the colonies affect the ability of the probe binding and whether to determine how smearing of the colonies during growth and lysis affects the quality product probe. It would also be worthwhile to repeat the colony blot hybridization using a higher stringency, by performing the washes at 63°C.

Another possibility is to evaluate the effect of the plasmid on the strength and length of the signal generated. It could be possible that the signal generated by the plasmid yielded a strong signal when first exposed to the X-ray film, but degrades quicker than the DNA-probe signal, which would allow colony blot hybridization to be used as a method of screening. However, the reliability of data generated would be determined by ability to differentiate between the “fast” and “slow” degradation of the signal strength.

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